IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF	Kranich et al.
SERIAL NO.	US 10/593,259
FILED:	September 18, 2006
FOR:	Non-Glycosylated/Non-Glycosidic/Non-Peptidic Small Molecule PSGL-1 Mimetics for the Treatment of Inflammatory Disorders

DECLARATION

I, Dr. Remo Kranich, born on December 28, 1969 in Hennigsdorf, Germany, having a bachelor ("Diploma") of chemistry,
a citizen of the Federal Republic of Germany and residing at Hennigsdorfer Strasse 141N in D-13503 Berlin, Germany, declare as follows:

I am a fully trained chemist, having studied chemistry at the Technical University of Berlin, Germany, from 1990 to 1996; I was awarded my bachelor ("Diploma") degree by the latter university in 1996; I finalized my PhD studies in organic chemistry at the Technical University of Berlin in 1999 and held a post-doctoral Biomedical Research Associate position at The Scripps Research Institute, La Jolla, CA, United States from 2000 until 2001.

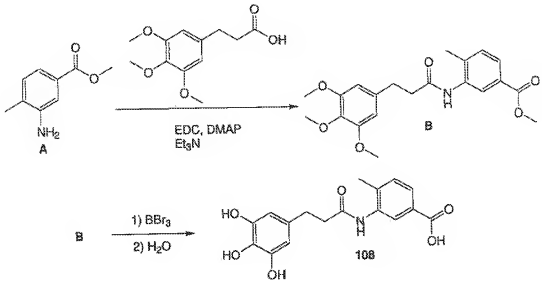
Since 2001, when I joined Revotar AG at Neuendorfstrasse 24a in D-16761 Hennigsdorf, Germany, I have been engaged in the synthesis, research and development of small molecule compounds.

I am one of the inventors of the invention disclosed and claimed in Application US 10/593,259 (filed September 18, 2006), and I am therefore fully conversant with the technical area to which application 10/593,259 pertains;

I have read the application and studied the application file, in particular the Office Action dated December 11, 2009, and the prior art referenced therein, and I am therefore also well acquainted with the invention, which is disclosed and claimed in US application 10/593,259.

The following experiments and tests were carried out under my supervision in accordance with the standardized procedures as described in the patent application and in particular on page 62 of the application. I have reviewed the test protocols and based on my review and knowledge, I consider those data to be fully reliable:

a) The following compound corresponding to Example **108** of the present patent application was prepared in our laboratories as following:

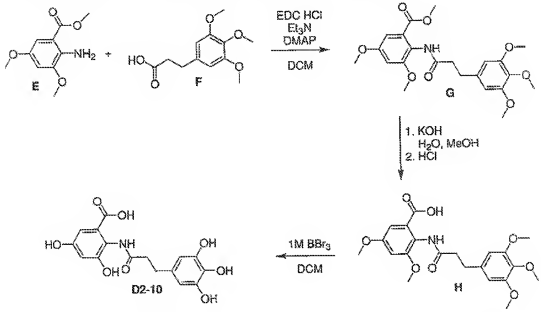


Step 1: (The following reaction is done in an anhydrous N_2 atmosphere.) Suspend EDC HCl (187 mg, 0.97 mmol) and Et_3N (136 µL, 0.97 mmol) in anhydrous dichloromethane (3.5 mL) and stir the resulting solution for 5 min at rt. Add 3-(3,4,5-Trimethoxy-phenyl)-propionic acid (234 mg, 0.97 mmol) and DMAP (12 mg, 0.1 mmol) and stir the resulting solution for 10 min. Add 3-Amino-4-methyl-benzoic acid methyl ester (**A**) (107 mg, 0.65 mmol) and stir the reaction solution overnight at rt. Quench reaction solution with water and saturated ammonium chloride solution, separate layers and extract aqu. layer several times with dichloromethane. Wash combined organic layer with brine, dry with Na_2SO_4, filtrate it through a short pad of silica gel using EtOAc/CyH (3+1) and remove solvent under reduced pressure. Purify the crude product by preparative radial chromatography (silica gel 60PF, CyH/EtOAc 1+1) to obtain 4-Methyl-3-[3-(3,4,5-trimethoxy-phenyl)-propionylamino]-benzoic acid methyl ester (**B**) (155 mg, 61%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): 2.11 (s, 3 H); 2.69 (t, 2 H, J = 7.2 Hz); 3.01 (t, 2 H, J = 7.2 Hz); 3.90 (br.s, 9 H); 3.88 (s, 3 H); 6.46 (s, 2 H); 6.77 (br.s, 1 H); 7.21 (d, 1 H, J = 8.1 Hz); 7.74 (br.d, 1 H, J = 7.6 Hz); 8.35 (br.s, 1 H).

Step 2: (The following reaction is done in an anhydrous N_2 atmosphere.) Dissolve 4-Methyl-3-[3-(3,4,5-trimethoxy-phenyl)-propionylamino]-benzoic acid methyl ester (**B**)

(103 mg, 0.26 mmol) in anhydrous dichloromethane (1.3 mL), cool the solution to –78°C and add dropwise BBr_3 (330 µL, 3.48 mmol). Stir the reaction mixture for 30 min at –78°C and after slowly warming up for additional 3.5 h at rt. Add dropwise ice-water followed by EtOAc, separate layers and extract aqu. layer several times with EtOAc. Wash combined organic layer with brine, dry with Na_2SO_4, filtrate and remove solvent under reduced pressure. Purify the crude product by preparative RP HPLC (gradient, water/CH_3CN 95:5 to 5:95) to obtain 4-Methyl-3-[3-(3,4,5-trihydroxy-phenyl)-propionylamino]-benzoic acid (**108**) (44 mg, 49%) as a white solid. ^1H NMR (400 MHz, CD_3OD): 2.21 (s, 3 H); 2.69 (t, 2 H, J = 7.4 Hz); 2.85 (t, 2 H, J = 7.4 Hz); 6.31 (s, 2 H); 7.34 (d, 1 H, J = 7.8 Hz); 7.81 (dd, 2 H, J_1 = 7.8 Hz, J_2 = 1.3 Hz); 8.01 (d, 1 H, J = 1.3 Hz).

b) The following compound **D2-10** corresponding to the prior art publication of Blaakmeer et al. in Journal of Natural Products, Volume 57, (8), pages 1145-1151, of 1994 was prepared in our laboratories by the following synthetic route:



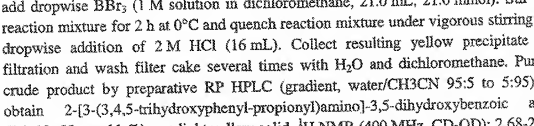
Step 1: (The following reaction is done in an anhydrous N_2 atmosphere.) Suspend EDC hydrochloride (1.63 g, 8.52 mmol) and Et_3N (1.20 mL, 8.52 mmol) in anhydrous dichloromethane (30 mL) and stir the resulting solution for 5 min at rt. Add 3-(3,4,5-trimethoxyphenyl)-propionic acid (**F**, 1.43 g, 5.97 mmol) and DMAP (104 mg, 0.85 mmol) and stir the resulting solution for 10 min at rt. Add methyl-2-amino-3,5-dimethoxybenzoate (**E**, 1.20 g, 5.68 mmol) and stir the reaction mixture 3 d at rt. Quench reaction solution with water (30 mL), separate layers and extract aqu. layer several times with dichloromethane. Wash the combined organic layer with water and

several times with dichloromethane. Wash the combined organic layer with water and brine, dry with Na_2SO_4 and remove solvent under reduced pressure. Dissolve crude product in EtOAc, filtrate over a short pad of silica gel and purify residue by column chromatography (silica gel 60, EtOAc/CyH 3+1, later 1+1, later 1+3) to obtain 2-[3-(3,4,5-trimethoxyphenylpropionyl)amino]-3,5-dimethoxybenzoic acid methyl ester (**G**, 747 mg, 30%) as a colorless oil. ^{1}H NMR (400 MHz, CD_3OD): 2.71 (t, 2 H, J = 7.6 Hz); 2.97 (t, 2 H, J = 7.6 Hz); 3.77 (s, 3 H)); 3.82 (s, 3 H); 3.84 (s, 3 H); 3.86 (br.s, 6 H); 3.87 (s, 3 H); 6.61 (s, 2 H); 6.82 (d, 1 H, J = 2.3 Hz); 6.94 (d, 1 H, J = 2.3 Hz).

Step 2: Dissolve 2-[3-(3,4,5-trimethoxyphenylpropionyl)amino]-3,5-dimethoxybenzoic acid methyl ester (**G**, 747 mg, 1.72 mmol) in a mixture of MeOH (9 mL) and H_2O (9 mL) at rt and add fine powdered KOH (400 mg, 7.12 mmol). Stir reaction mixture overnight at rt. Quench reaction mixture by addition of 1 M aqu. HCl (8.50 mL, 8.50 mmol). Collect the precipitate via vacuum filtration and wash the filter cake with water until a neutral filtrate is obtained. Dry filter cake in oil pump vacuum to obtain 2-[3-(3,4,5-trimethoxyphenylpropionyl)amino]-3,5-dimethoxybenzoic acid (**H**, 636 mg, 88%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD): 2.67-2.73 (m, 2 H); 2.94-3.00 (m, 2 H); 3.77 (s, 3 H)); 3.83 (s, 3 H); 3.86 (s, 6 H); 3.87 (s, 3 H); 6.61 (br.s, 2 H); 6.81 (d, 1 H, J = 2.8 Hz); 7.02 (d, 1 H, J = 2.8 Hz).

Step 3: (The following reaction is done in an anhydrous N_2 atmosphere.) Dissolve 2-[3-(3,4,5-trimethoxyphenylpropionyl)amino]-3,5-dimethoxybenzoic acid (**H**, 572 mg, 1.36 mmol) in anhydrous dichloromethane (14 mL), cool the solution to –78°C and add dropwise BBr_3 (1 M solution in dichloromethane, 21.0 mL, 21.0 mmol). Stir the reaction mixture for 2 h at 0°C and quench reaction mixture under vigorous stirring by dropwise addition of 2 M HCl (16 mL). Collect resulting yellow precipitate by filtration and wash filter cake several times with H_2O and dichloromethane. Purify crude product by preparative RP HPLC (gradient, water/CH3CN 95:5 to 5:95) to obtain 2-[3-(3,4,5-trihydroxyphenyl-propionyl)amino]-3,5-dihydroxybenzoic acid (**D2-10**, 55 mg, 11 %) as a light yellow solid. ^{1}H NMR (400 MHz, CD_3OD): 2.68-2.73 (m, 2 H); 2.83-2.88 (m, 2 H); 6.30 (s, 2 H); 6.55 (d, 1 H, J = 2.8 Hz); 7.02 (d, 1 H, J = 2.8 Hz).

The above applied intermediate **E** is obtained by the following route:



Step 1: (The following reaction is carried out in an N_2 atmosphere.) Dissolve methyl-3,5-dimethoxybenzoate (**C**, 9.26 g, 47.2 mmol) in Ac_2O (94 mL) at 8°C. Add

concentrated HNO_3 (9.49 mL), maintain reaction solution between 8°C and 15°C and stir mixture for 1 h at this temperature. Hydrolyze reaction mixture by addition of H_2O (100 mL), collect the resulting precipitate by vacuum filtration and wash filter cake with H_2O (3 x 50 mL). Purify resulting product by column chromatography (silica gel 60, CyH/EtOAc 1+1) to obtain methyl-2-nitro-3,5-dimethoxybenzoate (**D**, 3.05 g, 27%) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): 3.81 (s, 3 H); 3.89 (s, 3 H); 3.91 (s, 3 H); 6.97 (d, 1 H, J = 2.5 Hz); 7.07 (d, 1 H, J = 2.5 Hz).

Step 2: (The following reaction is carried out in an N_2 atmosphere.) Dissolve methyl-2-nitro-3,5-dimethoxybenzoate (**D**, 3.04 g, 12.6 mmol) in MeOH (180 mL) and add palladium on activated charcoal (1.34 g, 10 mol%, 10% Pd loading) and ammonium formate (7.94 g, 126 mmol). Degas the reaction mixture carefully (3 times), flush with N_2 again and stir for 45 min at rt. Filtrate mixture over a short pad of Celite and remove solvent under reduced pressure. Dissolve crude product in EtOAc and purify by filtration over a short pad of silica to obtain methyl-2-amino-3,5-dimethoxybenzoate (**E**, 2.53 g, 95%) as an off white solid. ^{1}H NMR (400 MHz, $CDCl_3$): 3.76 (s, 3 H); 3.85 (s, 3 H); 3.88 (s, 3 H); 6.58 (d, 1 H, J = 2.5 Hz); 6.93 (d, 1 H, J = 2.5 Hz).

c) The following in-vitro testing was performed:

Sialyl Lewis^X Tyrosine Sulfate Assay (sLe^x TSA):

Compounds of the present invention are assayed on a molecular level for their ability to inhibit the binding of P-, L-, or E-selectin chimeric molecules to sLe^x and tyrosine-sulphate residues linked to a polymeric matrix as a PSGL-1 substitute. Selected 50% inhibitory concentrations (IC_{50}-values) are determined. Microtiter plates are coated overnight in carbonate buffer pH 9.6 with goat anti human Fc mAB (10 µg/mL). After washing in assay buffer (25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 1 mM $CaCl_2$ pH 7.4) and blocking (3% bovine serum albumin (BSA) in assay buffer) plates are incubated for 2 h at 37°C with human P-Selectin-IgG-chimera (0.61 nM respectively 150 ng/mL) or human L-Selectin-IgG-chimera (0.61 nM respectively 89 ng/mL) or human E-Selectin-IgG-chimera (0.61 nM respectively 131 ng/mL). 5 µL of sLe^x -tyrosine sulfate polyacrylamide (1 mg/mL) carrying 15% sLe^x, 10% Tyrosine-sulphate and 5% biotin is complexed with 20 µL Streptavidin-Peroxidase solution (1 mg/mL) and 25 µL assay buffer without $CaCl_2$. For use in the assay, the ligand complex is diluted 1:10000 in assay buffer and further diluted 1:1 with varying amounts of compounds in assay buffer incl. 2% DMSO. This mixture is added to the wells precoated with E- or P-selectin. After incubation for 2 h at 37°C, wells are washed for six times with in assay buffer incl. 0.005%

Polyoxyethylenesorbitan monolaurate (TWEEN 20), developed for 10-15 min with 20 µL 3,3',5,5'-tetramethyl-benzidine (TMB)/H_2O_2 substrate solution and stopped with 20 µL 1M H_2SO_4. Bound sLe^X -Tyrosine sulphate ligand complex is determined by measuring optical density at 450 nm vs. 620 nm in a Fusion alpha-FP reader (sold by Packard Bioscience, Dreieich, Germany).

As the following Table 1 shows, the compound of the present invention (**108**) shows a more than 20fold higher ability of inhibiting all 3 selectins (E-, P-, and L-) than the compound **D2-10** of prior art document D2 (Blaakmeer et al.).

Results from sLe^XTSA: IC_{50} Data for E-/ P-/ L-Selectin:

Table 1

Compound	IC_{50} E-Selectin [µM]	IC_{50} P-Selectin [µM]	IC_{50} L-Selectin [µM]
108	1.3	1.7	1.8
D2-10	41	35	39

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.

By

Dr. Remo Kranich Date: April 28, 2010